# Carbohydrate production by phytoplankton and degradation in the marine microbial food web

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# Chapter 4

## Characterization of marine bacteria and the activity of their enzyme systems involved in degradation of the algal storage glucan laminarin

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**The algal storage glucan laminarin is one of the most abundant carbon sources for marine prokaryotes. Its degradation was investigated in bacteria isolated during and after a spring phytoplankton bloom in the coastal North Sea. On average, 26% of prokaryotes detected by epifluorescence counts were able to grow in Most Probable Number (MPN) dilution series on laminarin as a sole carbon source. Several bacterial strains were isolated from different dilutions and phylogenetic characterization revealed that they were belonging to different phylogenetic groups. The activity of the laminarin degrading enzyme systems was further characterized in three strains of *Vibrio* sp. that were able to grow on laminarin as a sole carbon source. At least two types of activity were detected upon degradation of laminarin: the release of glucose and the release of glucans larger then glucose. At saturating substrate concentrations the rate of glucan release exceeded the rate of glucose release, resulting in accumulation of glucan intermediates. At low concentrations, however, the ratio glucose/ glucan release increased, as a result of a higher affinity of the glucose releasing enzymes than of the glucan releasing enzymes. Therefore, at low substrate concentrations no accumulation of glucan intermediates occurred. The expression of laminarinase activity was depended on the presence of the substrate, and was repressed by the presence of glucose. In addition, low levels of activity were expressed under starvation conditions. Laminarinase enzymes showed a minimal activity on substrates with similar glucosidic bonds, but different sizes and secondary and/or tertiary structure. The characteristics found in these enzyme systems may help to elucidate factors hampering rapid carbohydrate degradation by prokaryotes.**

*Submitted*

## INTRODUCTION

Microbial communities in marine systems play a key role in the cycling of organic carbon and nutrients. An estimated 50% of the primary production is cycled as dissolved organic carbon (DOC) through the microbial loop to higher trophic levels (Azam 1998). Most of the bio-available DOC is present as high molecular weight (HMW) molecules (Amon and Benner 1994; Amon and Benner 1996) and has to be cleaved by extracellular enzymes prior to uptake, since bacteria can only transport substrates with a maximum molecular weight of approximately 600 Daltons through their cytoplasm membrane (Weiss et al. 1991). Based on the location of the extracellular enzymes, Chróst (1991) distinguished 'free' extracellular enzymes that occur dissolved in water or attached to surfaces other than the cell that produced them, and 'ectoenzymes', that cross the cytoplasm membrane and remain associated with the producing cell.

Polysaccharides are important constituents of HMW organic matter produced by algae (Biddanda and Benner 1997; Biersmith and Benner 1998). They display a remarkable structural diversity as a consequence of the wide variety of monosaccharides and the different glycosidic bonds between them. The primary structure is determined by the type of monosaccharides and their linkage, which leads to a secondary structure, determining the shape of the polysaccharides (e.g. ß-1,3-linked glucans form helices). Polysaccharides may be linked to each other by hydrogen bridges, determining a tertiary structure, e.g. a loose hydrogel, or a tightly packed network structure like cellulose.

Polysaccharides are degraded by glycoside hydrolases (EC 3.2.1.-), enzymes hydrolysing the glycosidic bond between 2 or more carbohydrate moieties. Based on the site of cleavage, enzymes can be classified as exo-acting enzymes that remove one or more sugar units from the end of a polysaccharide chain, and endo-acting enzymes that randomly hydrolyze bonds within the chains, thereby producing more ends for the exo-enzymes to act upon. Often a synergistic action of these different hydrolases is necessary for efficient degradation of polysaccharides (Driskill et al. 1999). Therefore degradation of a single substrate requires a carefully coordinated expression of the different enzymes, referred to as a system (Warren 1996).

The polysaccharide laminarin, the storage glucan found in many macro-algae and most phytoplankton, is one of the most abundant carbohydrates in the marine ecosystem. It is a soluble ß-1,3-D-glucose polymer with some branching at positions C-2 and C-6. The size typically ranges from 20-30 glucose residues. It is also known as laminaran, leucosin, or chrysolaminaran. In laminarin some chains are terminated by mannitol end-groups, which are not present in chrysolaminaran (Meeuse 1962; Painter 1983; Read et al. 1996). Chrysolaminaran is the principal storage glucan in diatoms and in the cosmopolitan genus *Phaeocystis,* which are both important phytoplankton groups driving global geochemical cycles (Nelson et al. 1995; Schoemann et al. 2005). Photosynthesis by diatoms alone generates as much as 40% of the 45 to 50 billion metric tons of organic carbon produced each year in the sea (Nelson et al. 1995). Glucan can account for up to 80% of the organic carbon of diatoms and *Phaeocystis* (Meeuse 1962; Myklestad 1974; Janse et al. 1996; Granum et al. 2002; Alderkamp et al. 2006). Therefore, an estimated 5-15 billion metric tons of chrysolaminaran are produced annually. Chrysolaminaran is located intracellular, in vacuoles (Chiovitti et al.

2004). It may be released as dissolved organic carbon (DOC) into the marine environment after algal cell lysis (Brussaard et al. 1995), or 'sloppy feeding' by copepods (Møller et al. 2003), where it will be one of the most abundant substrates for marine bacteria. Laminarin seems to rapidly degraded in the pelagic system (Keith and Arnosti 2001; Arnosti et al. 2005).

Very few studies have characterized enzyme systems of marine bacteria degrading substrates that are relevant in marine systems. Hydrolyzing activity in the marine environment has mainly been determined using small substrate proxies, consisting of a monomer such as glucose linked to a fluorophore such as methylumbelliferyl (MUF), whose fluorescence increases upon hydrolysis (e.g. Martinez et al. 1996; Arrieta and Herndl 2002). Since they lack the structural properties of real substrates, these substrate proxies will likely detect mainly exo-type of activities. Therefore, in this study laminarin was used as a relevant carbohydrate substrate to study the enzyme systems of marine bacteria that are abundant during a phytoplankton bloom in the coastal North Sea.

## MATERIALS AND METHODS

### Sampling

Surface water samples were collected from the Marsdiep, the Netherlands, during the phytoplankton spring bloom in 2002, from April through July. Samples were collected with a bucket, at high tide, two times a week. For chlorophyll *a* (Chl *a*) analysis, water samples were filtered onto Whatman GF/F filters, extracted in 90% (v/v) acetone and analysed fluorometrically according to Riegman et al. (1993). Phytoplankton abundance and species composition were determined on Lugol preserved samples under a Zeiss inverted microscope, using 5 ml counting chambers, as was described by Philippart et al. (2006). Total bacterial numbers were counted under an epifluorescence microscope after staining with Hoechst dye no. 33258 (Paul 1982) and by the Most Probable Number (MPN) technique (Clarke and Owens 1983). Marine medium described by Janse et al. (1999) was used in MPN dilutions, to obtain the fraction of culturable bacteria. To obtain bacteria able to grow on laminarin as a carbon source in MPN dilutions, 0.01% yeast extract (w/v, Becton Dickinson) and 0.01% casamino acids (w/v, Difco) of the marine medium was replaced by 2 mM glucose equivalents of laminarin from *Laminaria digitalis* (Sigma). Since laminarin is a natural substrate with variable polymer size, the substrate concentrations are expressed as glucose equivalents. All medium components were sterilized by autoclaving, except for the vitamins and the laminarin, which were filter-sterilized (0.2µm). The MPN counts were performed in 200µl medium in 250 µl, 96 well microplates, with 7 replicates, incubated at 12 ºC, for at least three weeks.

### Isolation of bacterial strains

Bacterial strains were isolated from the lowest and the highest positive MPN dilutions of the 29 June sample and from the highest positive dilution of the 15 July sample, by plating on solid medium consisting of the universal medium described above solidified by 2% agar (w/v, granulated, Becton Dickinson), incubated at 12 ºC. Bacterial cultures were grown in cotton

plugged Erlenmeyer flasks (culture volume <20% of the maximum Erlenmeyer volume), under continuous aeration (200 rpm), in the media described above, at 25 ºC.

**Sequencing of 16S rDNA gene**

Single colonies from plates were resuspended in sterile MilliQ water and used as templates in a PCR reaction using the universal 16S rDNA primers, B8F and U1406R (*E. coli*) (Sambrook et al. 1989). The amplified 16S rDNA gene was sequenced on an ABI automated DNA sequencer (PE applied biosystems) with primer U 1406 R. Sequence similarities for at least 500 bp of the 16s rDNA sequence, were determined by BLAST analysis (Altschul et al. 1997) of the National Center for Biotechnology Information database. Phylogenetic analysis of the obtained sequences and their close relatives was performed using the Neighbour Joining method with 1000 bootstrap replicates using MEGA version 3.0 software (Kumar et al. 2004).

**Preparation of extracellular and crude enzyme extracts**

Laminarin degrading activity was examined in pure cultures of bacteria grown on 2 mM laminarin as a sole carbon source. 200 ml of culture was harvested in mid-exponential growth phase, by centrifugation at 3500 g for 30 min at 4 ºC. To obtain cellular enzymes, including ectoenzymes, cell-pellets were washed twice with ice-cold artificial seawater buffered with Tris (pH 7.5), and resuspended in 50 mM sodium-phosphate buffer, pH 7. Cells were disrupted by French press (9000 bar) and debris was removed by centrifugation at 20,000 g for 10 min at 4 ºC. Since the cell debris interfered with the laminarinase assay and the supernatant contained more than 95% of the laminarin degrading activity, the supernatant was used as a crude extract of cellular and ectoenzymes. Extracts were stored on ice until the activity was assayed on the same day. To obtain extracellular enzymes, the supernatant from the harvested cultures was transferred to clean tubes and centrifuged again at 3500 g for 30 min at 4 ºC. The supernatant was stored on ice until the activity was assayed on the same day.

**Laminarinase assays**

Extracellular enzymes and crude cell extracts were tested for the capacity to hydrolyze laminarin. To the supernatant containing the extracellular enzymes, 10 mM glucose equivalents laminarin (final concentration) was added and triplicate samples were incubated at 25ºC. Crude cell extracts were diluted 1:10 in 50 mM sodium-phosphate buffer (pH 7.5) and 20 mM glucose equivalents of laminarin (final concentration) was added. Triplicate samples were incubated at 25ºC. After 3 h and overnight incubation a sample was taken, heat inactivated (3 min at 80ºC) and stored at -20ºC. The release of reducing sugar ends was measured according to Myklestad et al. (1997) and the release of glucose was measured using the Boehringer D-glucose test combination (Boehringer, Mannheim). The protein concentration was measured according to Bradford (1976). To test the effect of the buffer, incubations were also carried out using GF/P filtered and autoclaved natural seawater that was buffered with Tris (50 µM final concentration; pH 7.5).

**Kinetic analyses: apparent $K_M$ and $V_{max}$ determinations**

Apparent $K_M$ and $V_{max}$ were determined for crude extracts from each strain. Total activity and glucose release were determined at different substrate concentrations (0.1-20 mM glucose eq). At least eight substrate/ activity data pairs were fitted according to Michaelis-Menten kinetics using the non-linear regression program TableCurve (Jandel Scientific, AISN Software).

**Substrate specificity**

The substrate specificity of crude extracts was determined using the enzyme activity assay described above using 0.5% (w/v) of the following substrates: curdlan from *Alcaligenes faecalis* (Sigma), ß-glucan, dietary fiber control (Sigma), ß-D-glucan from barley (Sigma), lichenan from *Cetraria islandica* (Sigma), ß-1,3-glucan from *Euglena gracilis* (Fluka), and 20 mM glucose equivalents pullulan from *Aureobasidium pullulans* (Sigma). To determine the solubility of these substrates, solutions were incubated at room temperature for 1 hour and mixed several times, before centrifugation (14,000 g, 10 min). Total carbohydrate concentration was determined in the supernatant by the phenol sulphuric acid method (Liu et al. 1973) .

## RESULTS AND DISCUSSION

**Isolation of bacterial strains from MPN dilution series growing on laminarin as a sole carbon source**

The phytoplankton bloom of 2002 was dominated by the colony forming Prymnesiophyte *Phaeocystis globosa* from 6 June until 27 June. Prokaryote numbers varied between 2.3 x $10^9$ and 3.3 x $10^9$ cells $l^{-1}$ over the period April through July (Figure 1). On average, 26% of the prokaryotes detected by epifluorescence microscopy were able to grow in the MPN dilution series, both on universal medium and on laminarin medium. The percentage of prokaryotes that were able to grow on the universal medium increased from 10-12% during the wax of the *P. globosa* bloom to 73 % during the wane of the bloom (29 June). The percentage of prokaryotes able to grow on laminarin was highest (66%) on 5 June and varied between 4-28% in the other samples.

Nineteen bacterial strains were isolated from several dilutions of the MPN series on laminarin as a sole carbon source and subjected to phylogenetic analysis (Table 1, Figure 2). Strains isolated from the same dilution with identical 16 S rDNA sequences were considered to be the same (numbers in brackets in Table 1). The isolates affiliated with different phylogenetic groups that are known to be abundant in coastal waters, such as *Roseobacter*, Bacteroidetes, *Pseudoalteromonas,* and *Vibrio* (Eilers et al. 2000b; Pinhassi et al. 2004). Therefore, the capability of degradation of laminarin is not restricted to a single group of prokaryotes, but seems to be a common feature amongst marine bacteria. Since the strains isolated from the highest dilutions are abundant in the sea, they are likely to play a role in degradation of laminarin in the sea.

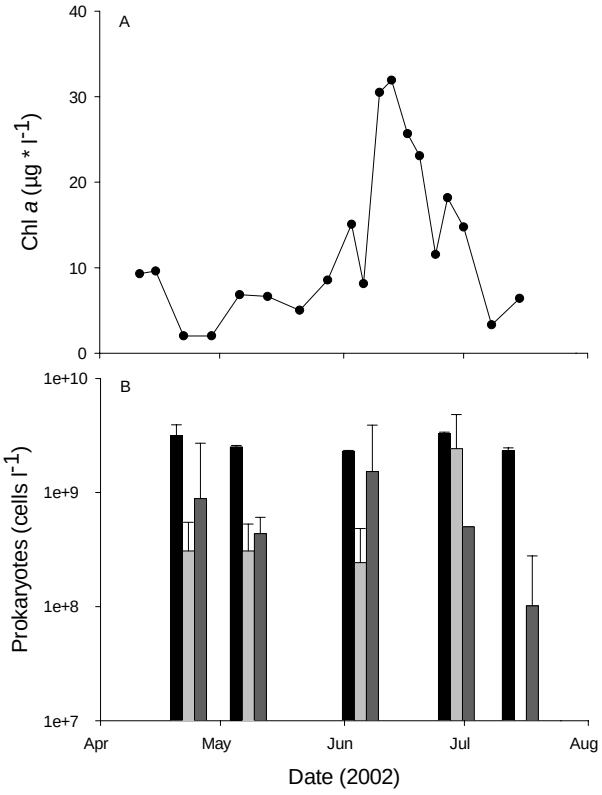


**Figure 1.** (A) Temporal dynamics of chlorophyll *a* during spring and summer 2002. (B) Total prokaryote numbers counted by epifluorescence microscopy (black bars) and the Most Probable Number (MPN) technique on "universal medium" (light grey bars) and medium containing laminarin as a sole carbon source (dark grey bars). Data of MPN counts on universal medium on 15 July are missing due to an infection. Error bars indicate standard deviation of at least 10 counted fields (microscopy), or 95% confidence interval of 7 replicates (MPN).

Members of *Roseobacter* and Bacteroidetes have previously been isolated from the coastal North Sea, and culture independent analysis showed their abundance in marine systems (Eilers et al. 2000a; Eilers et al. 2000b). In addition, they were detected during and after a *Phaeocystis* bloom in a mesocosm (Brussaard et al. 2005b) and in stable microbial enrichments degrading *Phaeocystis* carbohydrates (Janse et al. 2000). Gamma-proteobacteria such as *Pseudoalteromonas* and *Vibrio* have also frequently been isolated, but usually comprise < 1 % of the total prokaryote population (Eilers et al. 2000a, 2000b). Yet, bacteria from the genus *Vibrio* are ubiquitous and have long served as models for heterotrophic processes. They play an important role in coastal seas and estuaries owing to their widespread abundance and high metabolic activities. They are present both as free-living bacteria and attached to particles, algae, copepods and fish (Huq et al. 1990; Heidelberg et al. 2002). They are capable of grow-

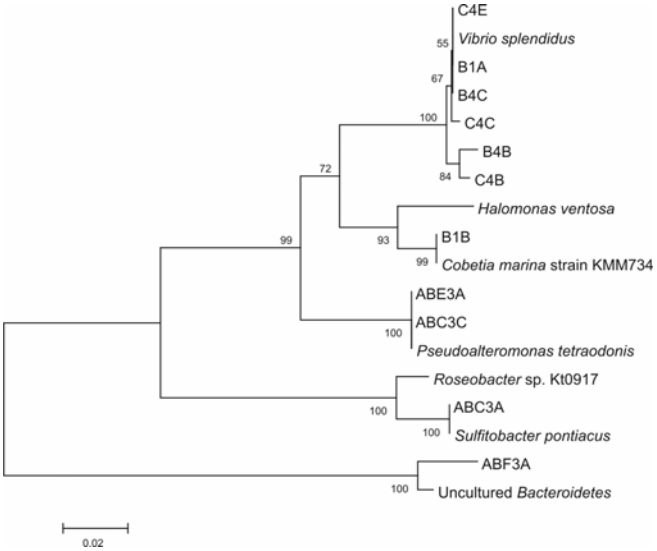


**Figure 2.** Neighbor-joining tree based on partial 16S rDNA sequences derived from bacterial isolates and close relatives (identified via BLAST search). The scale bar indicates 2% of sequence variation.

**Table 1.** Bacterial strains isolated from the MPN dilution series on laminarin as a sole carbon source inoculated with surface samples from the Marsdiep, the Netherlands

| **Strain** | **Sample date** | **Sample dilution** | **Presence in original sample (cells $l^{-1}$)** | **Closest phylogenetic match** |
|---|---|---|---|---|
| B1A | 29 June | 1:10 | 5 x $10^4$ | *Vibrio splendidus* |
| B1B (4)[1] | 29 June | 1:10 | 5 x $10^4$ | *Cobetia marina* |
| B4C | 29 June | 1: $10^4$ | 5 x $10^7$ | *Vibrio splendidus* |
| C4B | 29 June | 1: $10^4$ | 5 x $10^7$ | *Vibrio* sp. PMV19 |
| C4C | 29 June | 1: $10^4$ | 5 x $10^7$ | *Vibrio* sp. PMV19 |
| C4E | 29 June | 1: $10^4$ | 5 x $10^7$ | *Vibrio splendidus* |
| ABE3A (2) | 15 July | 1: $10^3$ | 5 x $10^6$ | *Pseudoalteromonas tetradonis* |
| ABC3C (2) | 15 July | 1: $10^3$ | 5 x $10^6$ | *Pseudoalteromonas tetradonis* |
| ABC3A (5) | 15 July | 1: $10^3$ | 5 x $10^6$ | *Sulfitobacter pontiacus* |
| AB F3A | 15 July | 1: $10^3$ | 5 x $10^6$ | Uncultured member of *Bacteroidetes* |

[1] in brackets are the number of strains isolated from the same dilution sample with identical 16 S rDNA sequence

ing rapidly under nutrient-rich conditions and surviving prolonged periods of starvation (Oliver et al. 1991; Nyström et al. 1992; McDougald et al. 2002) and are known to grow on complex substrates such as chitin (Meibom et al. 2004; Li and Roseman 2004). Since members of the genus *Vibrio* were isolated both from enrichments and from the highest dilution, we chose three *Vibrio* strains for further characterization of enzymes involved in the degradation of laminarin.

**Laminarinase activity in three strains of *Vibrio* sp.**

The laminarinase activity was not affected by the use of either Tris-buffered, filtered seawater or sodium phosphate buffer, therefore the sodium phosphate buffer was used in the assays. Laminarinase activity was detected both in the medium and in crude cell extracts of the three *Vibrio* strains, indicating the presence of both extracellular and ectoenzymes. Since more than 90% of the activity could be detected in the crude cell extract, this was used for further characterization of the enzyme systems degrading laminarin. These extracts likely include intracellular, periplasmatic and/or ectoenzymes. The total laminarinase activity closely followed Michelis-Menten kinetics in each of the three strains ($r^2$ = 0.978, 0.943, 0.944 respectively). At least two types of activity were detected: release of glucose and release of glucans larger then glucose (Table 2). These activities are consistent with the presence of a gene encoding an endo-ß-glucanase of the family 16 (EC 3.2.1.39) and genes encoding exo-ß-1,3 glycosidases of the family 17 (EC 3.2.1.58) in the genome sequence of *Vibrio vulnificus* (Kim et al. 2003). Since substrate cleavage by an endo-ß-1,3-glucanase yields a new free end where the exo-ß-1,3 glycosidase can act upon, the synergistic interaction of these enzymes is likely to be responsible for an efficient degradation of laminarin. Upon prolonged incubation, laminarin was degraded for more than 95% to glucose by the crude extracts of each of the three strains.

Both the total laminarinase activity ($V_{max}$) and the affinity constant ($K_m$) were highest in strain B1A and similar in B4C and C4B (Table 2). The $V_{max}/K_m$ ratio, which represents the slope of the Michaelis-Menten equation at low substrate concentrations, is an indicator of the

**Table 2.** Apparent kinetic parameters for laminarinase activity of crude cell extracts of *Vibrio* sp. strains B1A, B4C, and C4B. One unit of activity is expressed as µmol reducing ends released per hour per gram protein at pH 7.5 and 25 ºC. In brackets is the standard deviation (S.D.) of at least eight substrate/ activity data pairs.

| Strain | Total activity $V_{max}$ (U) | Total activity $K_m$ (mM) | Total activity $V_{max}/K_m$ | Glucose releasing activity $V_{max}$ (U) |
|---|---|---|---|---|
| B1A | 34.17 (1.84) | 4.50 (0.73) | 7.6 | 0.83 (0.03) |
| B4C | 10.26 (0.67) | 0.78 (0.16) | 13.1 | 0.90 (0.02) |
| C4B | 8.53 (0.36) | 0.57 (0.12) | 15.0 | 0.81 (0.03) |

ability of the strain to obtain high hydrolysis rates at low substrate concentrations (Healey 1980). This ratio was for strain B1A higher than for strains B4C and C4B, suggesting that strain B4C and C4B are better competitors at low substrate concentrations, whereas strain B1A would be a better competitor at higher substrate concentration.

The glucose releasing activity was approximately 10% of the total laminarinase activity at saturating substrate concentrations (Table 2). The higher rate of glucan release than that of glucose resulted in the accumulation of glucan intermediates during degradation at high substrate concentrations (Table 3). At lower substrate concentrations, however, the proportion of reducing ends released as glucose increased, suggesting a lower $K_m$ value for the glucose releasing activity than for the total activity. Release of glucans was also reported during microbial degradation of high concentrations (2 % w/v) of complex carbohydrates in *Laminaria* thallus (Uchida 1995). In general, bacterial hydrolysis of polymers of aggregates and uptake of low molecular weight compounds may be uncoupled processes, resulting in release of free polymers from particles into the surrounding water mass (Cho and Azam 1988; Smith et al. 1992; 1995; Unanue et al. 1998; Azúa et al. 2003). In aggregates, the carbohydrate concentrations are high (Azúa et al. 2003), leading to high substrate concentrations for glucosidases. If the differences in kinetic properties between the release of glucan and glucose are exemplary for other endo-hydrolase and exo-hydrolase activities, the higher $V_{max}$ of endo-hydrolases will lead to the release of polymers from particles. Since the ratio glucose/ glucan release increased at lower substrate concentrations (Table 3), accumulation of glucan intermediates is unlikely to occur in the environment outside aggregates, where substrate concentrations are much lower.

To compare the kinetic parameters with those of different ß-glucosidases present during and after a bloom of *P. globosa* in the coastal North Sea, determined using the fluorogenic substrate analogue MUF-beta-D-glucoside (Arrieta and Herndl 2002), we express the $K_m$ values per mol substrate using an average size for the laminarin molecule of 25 glucose units. This leads to $K_m$ values for the total activity of 180, 31.2 and 22.8 µmol $l^{-1}$ laminarin for strains B1A, B4C, and C4B respectively. These values are in the range of 12.1 to over 282.7 µmol $l^{-1}$ MUF-glucose detected by (Arrieta et al. 2002).

**Table 3.** The ratio of glucose to glucan formation after overnight incubation of crude cell extracts of *Vibrio* sp. strains B1A, B4C, and C4B with different concentrations of laminarin.

| **Strain** | **Laminarin concentration** | | |
|---|---|---|---|
| | **1 mM** | **5 mM** | **10 mM** |
| B1A | 0.85 | 0.18 | 0.11 |
| B4C | 0.85 | 0.15 | 0.14 |
| C4B | 0.67 | 0.18 | 0.11 |

**Expression of laminarinase activity in three strains of *Vibrio* sp.**

When cultures were grown on glucose or pyruvate as a sole carbon source, no laminarinase activity was detected in crude extracts (Table 4). When cultures were grown on a mixture of laminarin and pyruvate as carbon sources, low levels of laminarinase activity were detected. Therefore, we conclude that during the exponential growth phase of the *Vibrio* strains, expression of laminarinase activity was dependent on the availability of laminarin. However, when cultures were grown on a mixture of laminarin and glucose, no laminarinase activity was detected. This suggests that synthesis of laminarinase is repressed in the presence of glucose. In the stationary growth phase, laminarinase activity was detected in all cultures. Stationary cultures grown on either pyruvate or glucose expressed low levels of laminarinase activity, whereas cultures grown on a mixture of laminarin and glucose, or laminarin and pyruvate expressed an intermediate activity.

Enzyme synthesis triggered by the presence of a suitable substrate and inhibition by monomeric compounds is a common feature of beta-glucosidases in marine bacteria (Chróst 1991; Middelboe et al. 1995). The expression of low levels of activity upon carbon starvation resembles the expression of extracellular chitinase activity upon starvation in *Vibrio furnisii* (Bassler et al. 1991; Li and Roseman. 2004). The explanation put forward by Li and Roseman, is that secreted chitinase from starving cells comes into contact with the insoluble chitin in the microenvironment of the *Vibrio* and generates a disaccharide and/or oligomer gradient. The *Vibrio* senses the soluble oligomer intermediates and swims up the gradient towards the chitin. In addition oligomers induce the expression of the full chitin degradation system. Although laminarin is a soluble substrate, and may therefore directly be sensed by the *Vibrio* we speculate that expression of the laminarin degradation system may be regulated in a similar fashion. Thus, expression upon carbon starvation of different extracellular hydrolase enzymes may be a mechanism for the sensing of potential substrates in the *Vibrio* microenvironment.

**Table 4.** Relative laminarinase activity normalized to $V_{max}$ rates of crude extracts of cells grown on laminarin and harvested at mid exponential phase.

| Growth phase | Carbon- source | B1A | B4C | C4B |
|---|---|---|---|---|
| exponential | Laminarin | 100 % | 100 % | 100 % |
| | Pyruvate | ND | ND | ND |
| | Glucose | ND | ND | ND |
| | Pyruvate + laminarin | 22 % | 15 % | 11 % |
| | Glucose + laminarin | ND | ND | ND |
| stationary | Laminarin | 78 % | 132 % | 104 % |
| | Pyruvate | 3 % | 3 % | 2 % |
| | Glucose | 9 % | 3 % | 2 % |
| | Pyruvate + laminarin | 21 % | 29 % | 44 % |
| | Glucose + laminarin | 12 % | 32 % | 34 % |

**Substrate specificity of the laminarinase enzymes**

The activity of the laminarinase enzymes in the crude cell extracts of the *Vibrio* sp. strains grown on laminarin until mid-exponential phase was tested on several glucose polymers that differ from laminarin in size, solubility and structure (Table 5A). Curdlan and glucan from *Euglena gracilis* are both ß-1,3-glucans and thus have a similar primary and secondary structure to laminarin, but are much larger in size and are insoluble polymers. There was a low activity on curdlan, but no activity was detected on the glucan from *Euglena gracilis* (Table 5B). Barley glucan and lichenan have ß-1,3-glucosidic bonds, connecting stretches of ß-1,4-linked glucose and consequently differ in secondary and tertiary structure form laminarin. A low activity was detected on both substrates. Pullulan is a repeating structure of three α-1,4-linked glucoses (maltotriose) connected by α-1,6- glucosidic bonds, a soluble substrate differing from laminarin in its primary, secondary and tertiary structure, on which no activity was detected. Although each of the *Vibrio* strains was able to grow on pullulan as a sole carbon source (results not shown), the *Vibrio* strains apparently used other enzyme systems to degrade this substrate. The absence of pullulanase activity in strains grown on laminarin as a sole carbon source shows that expression of pullulanase activity is likely to be dependent on the presence of pullulan, in a similar way expression of laminarinase activity was dependent on the presence of laminarin.

**Table 5A.** Relevant information on the substrates used to determine the substrate specificity of crude cell extracts of *Vibrio* sp. strains B1A, B4C, and C4B grown on laminarin to mid exponential phase.

| Substrate | Backbone | Branches | Size | Solubility | Source |
|---|---|---|---|---|---|
| Laminarin | ß-1,3-glucose | ß-1,6 | 3.9 kD | 100 % | Food reserve in most algae |
| Barley glucan | ß-1,3 cellotriose and cellotetrose | no | 49 MD | 21.6 % | Cell wall constituent in Barley and other higher plants |
| Lichenan | ß-1,3-1,4 glucose | no | No info | 15.7 % | Cell wall constituent of Irish moss |
| Dietary glucan | No info | No info | No info | 5.5 % | |
| Curdlan | ß-1,3-glucose | no | 100 kD | 0.15 % | Extracellular bacterial glucan |
| *Euglena* glucan | ß-1,3-glucose | no | 500 kD | 0.12 % | Food reserve in yeast |
| Pullulan | α-1,6-maltotriose | no | 200 kD | 100 % | Extracellular polysaccharide in yeast, structurally similar to amylopectine in starch |

**Table 5B.** Relative activity of crude cell extracts of *Vibrio* sp. strains B1A, B4C, and C4B normalized to $V_{max}$ rates of crude extracts at mid exponential phase grown on laminarin.

| Strain | Barley glucan | Lichenan | Dietary glucan | Curdlan | *Euglena* glucan | Pullulan |
|---|---|---|---|---|---|---|
| B1A | 1.9 % | 2.4 % | 0.7 | 3.4 % | n.d. | n.d |
| B4C | 1.7 % | 1.1 % | n.d.* | 1.2 % | n.d. | n.d. |
| C4B | 0.7 % | 1.5 % | n.d. | 1.9 % | n.d. | n.d |

*n.d. no activity could be detected, the detection limit being 0.5% of the activity on laminarin

The minimal activity of laminarinase enzymes on substrates similar to laminarin with respect to their primary and secondary structure may have important implications for polymer degradation in the marine environment. Polymers derived from algae are known to selfassemble into hydrogels (Chin et al. 1998), that may be the precursor for larger particles, such as transparent exopolymeric particles (TEP) or marine snow (Verdugo et al. 2004). Although particles are regarded as "hotspots" of microbial abundance and activity (Azam 1998), assemblage may influence the secondary structure of the polymer, analogous to the difference between laminarin and curdlan. If the difference in degradation potential of laminarin versus curdlan is exemplary for the difference in degradation potential of "free" polymers and polymers embedded in a gel structure, turnover times may be increased from days to years. This may be an additional explanation why carbohydrates are usually regarded as labile substrates for marine micro-organisms, but nevertheless form an important fraction of the DOM in the marine environment (Benner et al. 1992), in marine sediments as well as sedimentary pore water (Cowie and Hedges 1984; Arnosti and Holmer 1999).

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